Effect of Temperature and Growth Media Composition on Kinetics of BLIS production by *Enterococcus faecalis* T23

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The aim of this research was to study the effects of temperature and nutrient media on the growth and bacteriocin-like inhibitory substance (BLIS) production by strain *E. faecalis* T23. The time course of BLIS production and growth kinetics were determined at 37, 30 and 45ºC in MRS media, also at 37ºC in M17 media and in M17 media supplemented with glucose (0,5% w/v). Optimal conditions for the growth and BLIS production for the studied strain were found to be 37ºC and M17 media supplemented with glucose. Due to BLIS production, *E. faecalis* T23 could represent a new adjunct culture for the dairy industry and could be used as natural bioconservant.

Key words: *lactic acid bacteria, bacteriocins, cheese*

INTRODUCTION

Lactic acid bacteria (LAB) is metabolically and physiologically related group of Gram-positive, catalase-negative, strictly fermentative bacteria, producing lactic acid as the major end product of sugar fermentation. Typical LAB are non-sporing and non-respiring, devoid of cytochromes, aero- and acid-tolerant and fastidious. The LAB include both non-pathogenic species that are used for industrial fermentation of dairy products, meats, and vegetables, and for the production of wine, silage and sourdough, and some pathogens, e.g., several *Enterococcus* species.

One of the main beneficial properties of LAB in food fermentation is their ability to produce antimicrobial compounds and thus prevent or inhibit the growth of food spoilage bacteria. These antimicrobial compounds could provide the safety of fermented food product and prolong the shelf life.

The antimicrobial compounds produced by LAB are lactic acid, hydrogen peroxide, diacetyl and bacteriocins. Bacteriocins are ribosomly synthesized peptides that have antibacterial activity, mainly towards closely related bacterial species (Klaenhammer, 1993; Nes et al., 1996). Bacteriocins can be either cell bound or released extracellularly and may be produced early or late in the growth cycle. They are susceptible to proteases and have variable stability at different pH and temperature.

Different classification schemes of bacteriocins were proposed over the last decade. First, Klaenhammer (1993), then Franz et al. (2007) with some modifications subdivide bacteriocins into four classes according to their biochemical properties: Class I, which corresponds to bacteriocins known as lantibiotics, Class II corresponds to low molecular mass < 10 kDa, heat stable and membrane-active peptides without modified amino acid residues, Class III cyclic antibacterial peptides; and Class IV, large proteins. Recently, Zouhir et al (2010) proposed a new structure based classification that regroups bacteriocins into 12 classes.

Enterococci play beneficial roles in foods due to production of powerful bacteriocins named enterocins (Sarantinopoulos et al., 2001; Giraffa, 2003; Foulquié-Moreno et al., 2006; Psoni et al., 2006). Enterocins represent antimicrobial compounds not only active against strains closely related to the producer micro-organisms but also displaying large spectra of inhibition against food-spoiling or pathogenic bacteria such as *Listeria* sp., *Staphylococcus aureus* or *Bacillus* sp (Hadji-Sfaxi et al., 2011). Therefore, enterocins have potential for use as food additives.

In the present work we studied the optimal conditions for the growth and bacteriocin-like inhibitory substance production for the strain *E. faecalis* T23. This strain is a producer of antimicrobial compound of protein nature, however as we didn't prove yet that this compound is bacteriocin, we named it bacteriocin-like inhibitory substance (BLIS).

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. Strain *Enterococcus faecalis* used in this study was isolated from traditional cheese obtained from individual household in Isfahan city of Iran Islam republic. Strain was reconstituted in sterile skim milk (12.5%, w/v) supplemented with 30% (w/v) glycerol and stored at –80° C. Before using strain was propagated twice in MRS media.

All media were supplied by Difco (Detroit, MI, USA).

Determination of growth, acidification and BLIS production kinetics. The time course of BLIS production was determined at 37, 30 and 45 ºC in MRS media, also at 37 ºC in M17 media and in M17 media supplemented with glucose (0.5% w/v). Nutrient media was inoculated with overnight cultures (2% v/v) and incubated under non regulated pH conditions. The pH decrease was measured by pH-meter. Antimicrobial activity (AU/mL) of the BLIS production, as well the modifications in pH and optical density (OD 600nm) of the cultures were determined at regular intervals (1 h) during 24 h. Antimicrobial activity in AU/mL was calculated according to spot-on-lawn method (Yamamoto et al., 2003). Cell-free supernatants of overnight (16-18 h) cultures were obtained by centrifugation (10000 x g, 15 min, 4° C) and pH was adjusted to 6.5 with 1N NaOH. To avoid proteolytic degradation of the bacteriocins, cell-free supernatants were treated for 10 min at 80º C. The resulting sample was serially diluted twofold with Na-phosphate (100 mM, pH 6.5) and each dilution was spotted on the lawn of agar media containing sensitive strain. Agar media with sensitive strain was prepared as follow: soft nutrient agar (0.8%, w/v) was solidified in a sterile Petri dish after addition of indicator strain culture, grown to the early stationary growth phase in nutrient medium. The plates were left to dry at the room temperature before spotting the different titrations of supernatants. The plates were incubated at 37°C overnight, and the titr was defined as the reciprocal of the highest dilution (2^n) that resulted in inhibition of the indicator lawn. Thus, the AU of antimicrobial activity per milliliter was defined as $2^n \times 1000$ µl 10 μL^{-1}. *Lactobacillus brevis* F145 was used as sensitive strain. All analyses were performed in duplicate.

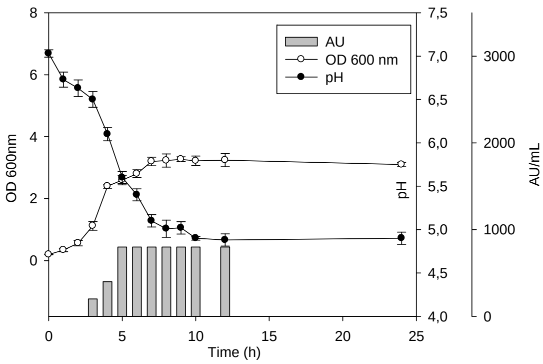
RESULTS AND DISCUSSION

In order to determine the optimal conditions for the growth and BLIS production by strain *E.faecalis* T23, we incubated the strain at different temperatures using three different media, known to be optimal for the growth of *Enterococci*. The time course of BLIS production was determined at 37, 30 and 45º C in MRS media, also at 37º C in M17 media and in M17 media supplemented with glucose (0.5% w/v).

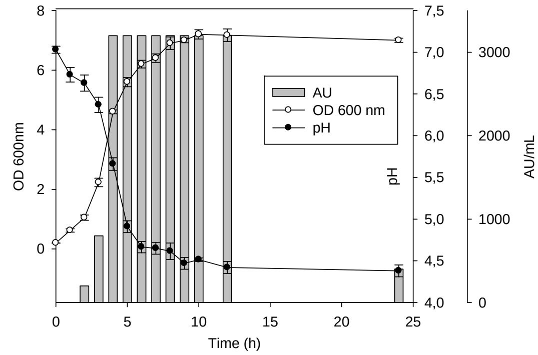
During the growth in MRS media the optimal temperature for BLIS production found to be 37º C (Fig. 1 b). During the growth at this temperature the strains exerted the good acidification level (pH low than 5.0) and also high growth level with cell density higher than 7.2 (OD 600nm). At this incubation temperature *E. faecalis* T23 started to produce bacteriocin (200 AU/mL) at 2 h growth point of logarithmic growth phase. Maximum production (3200 AU/mL) was reached at the end of the exponential phase (4 h) and remained stable till the late stationary phase. However it was decreased after 24 h (400 AU/mL). At 30º C the growth was less and very low activity level (max 800 AU/mL) was detected (Fig. 1 a). We can see from the graph that strain also started to produce BLIS at the beginning of exponential phase and reached the maximum level at the end of the stationary phase. However it is difficult to make conclusions, as the cell density was lower, than it was observed at 37º C. Thus, we can't conclude, if the strain produce lower amount of BLIS, or the low activity was due to the lower cell density.

At 45º C the strain grows faster and enters the exponential and stationary growth phase earlier (Fig. 1 c). It also started to produce BLIS (400 AU/mL) at 2 h growth point of logarithmic growth phase. Maximum production (3200 AU/mL) was reached at the end of the exponential phase (4h) and remained stable till the early stationary phase. However it was decreased in the middle of stationary phase (1600 AU/mL). After 24 h incubation no activity was detected. Thus, we can conclude that in MRS media the optimal temperature for bacteriocin production was 37º C, and for the growth 45º C. It seems that at the some point of cell density the stability of BLIS production was decreased. This phenomen might be due to different factors. One of the explanations is the possible inactivation of BLIS in the presence of some accumulated metabolites, which concentration increase according to the increase of the cell density.

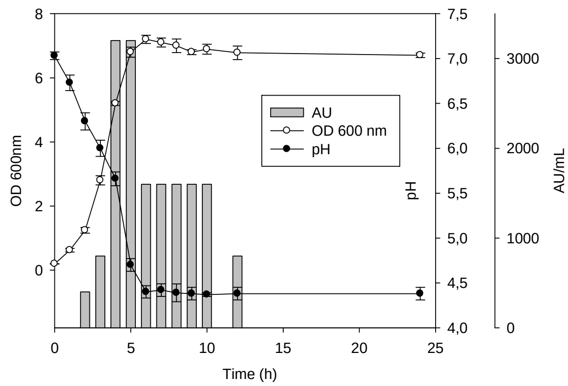
The ability of growth and BLIS production by *E. faecalis* T23 was also tested in M17 media which is known to be selective for cocci. The temperature of incubation was 37 ºC. As it is shown in the Fig. 2 a, when the simple M17 media was used for cultivation, the strain entered the exponential growth phase with some retard, compared to MRS media. The bacteriocin production was detected after 4 h growth and reached the maximum level at the beginning of stationary phase (3200 U/mL at 8 h growth point). When M17 media was supplemented with glucose, the bacteriocin production level was detectable already after 1 h of growth and strain enter very fast the exponential growth phase (Fig. 2 b). Maximum BLIS production was reached after 4 h growth (3200 AU/ mL and retained stable. After 24 h incubation bacteriocin activity was less (800 AU/mL).



a

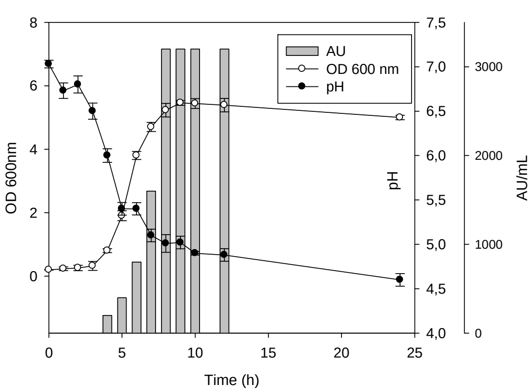


b



c

Figure 1. Kinetics of growth, acidification and BLIS production in MRS media at 30°C (**a**), 37°C (**b**) and 45° (**c**).



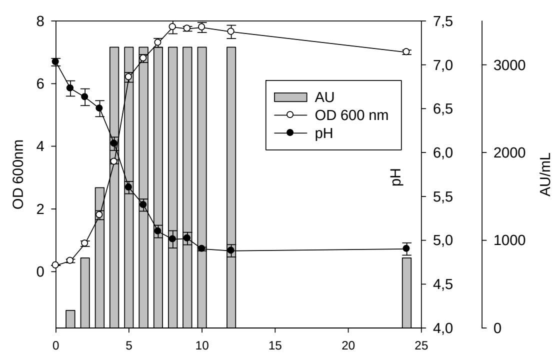


Figure 2. Kinetics of growth, acidification and BLIS production in M17 media (**a**) and M17 media supplemented with glucose (**b**) at 37°C.

Even this profile of bacteriocin production and growth is not very different from what was observed in MRS media at the same temperature, it is obvious that strain started to produce the antimicrobial agent faster and even after 24 h the level of bacteriocin activity was higher than it was observed in MRS media. Thus, we can conclude, that the optimal media for BLIS production by *E. faecalis* T23 is M17 supplemented with glucose.

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Enterococcus faecalis T23 Ştammının Bakteriosinəbənzər Ingibəedici Maddəsinin Sintezinə Temperaturun və Qida Mühitinin Təsiri

Tədqiqatın əsas məqsədi *Enterococcus faecalis* T23 ştammının ishal etdiyi bakteriosinəbənzər ingibəedici maddəyə müxtəlif faktorların təsirinin öyrənilməsi olmuşdur. Tədqiq olunan ştamm tərəfindən bakteriosinəbənzər ingibəedici maddənin sintezi, mühiti turşutma və böyümə kinetikası 37, 30 və 45º C də MRS mühitində, həmçinin 37º C də M17 və qlükoza əlavə edilmiş M17 mühitində tədqiq olunmuşdur. Tədqiq olunan ştamm tərəfindən bu maddənin sintezi üçün optimal şərait 37ºC temperatur və qlükoza əlavə edilmiş M17 qida mühiti olmuşdur. Ayrılmış fəal ştamm bakteriosinəbənzər ingibəedici maddənin produsentidir və qida sənayesində istifadə üçün potensiala malikdir.

Т.Б. Мирхади Зади, А.А. Кулиев

Влияние Температуры и Питательной среды на Синтез Бактериоциноподобного Ингибирующего Вещества Штаммом *Enterococcus faecalis* T23

Целью исследования было изучение влияния различных факторов на синтез бактериоциноподобного ингибирующего вещества штаммом *Enterococcus faecalis* T23. Кинетика продукции бактериоциноподобного ингибирующего вещества, роста и подкисления среды исследуемым штаммом была изучена при росте в MRS среде при температурах 37, 30 и 45º C, а также при температуре 37º C в M17 среде и в модифицированной M17 среде, в которую добавляли глюкозу. Было определено, что оптимальными условиями для синтеза бактериоциноподобного вещества штаммом *Enterococcus Faecalis* T23 являются температура 37ºC и модифицированная M17 среда, в которую добавляли глюкозу. Полученный активный штамм является продуцентом бактериоциноподобного ингибирующего вещества и обладает потенциалом использования в пищевой промышленности в качестве стартерной или добавочной культуры.